MATERIALS AND METHODS

3.1 Media

3.1.1 Nutrient broth (NB)

Peptone : 0.5%

NaCl : 0.5%

Meat Extract : 0.15%

Yeast Extract : 0.15%

pH : 7.0 ± 0.2

3.1.2 Minimal media

Table 3.1: Composition of minimal media for xylanase production.

Minimal Media (per liter)	
K_2HPO_4	1.5 g
KH_2PO_4	3.0 g
$MgSO_4.7\,H_2O$	0.3 g
$CaCO_3.2\,H_2O$	0.05 g
NaCl	0.5 g
$NaHCO_3$	0.5 g
NH_4Cl	1.0 g
$FeSO_4.7H_2O$	1mg
NH_4Fe(III) citrate	10 mg
$MnSO_4.H_2O$	5 mg
$CoCl_2.6H_2O$	1mg
$ZnSO_4.7H_2O$	1 mg
$CuSO_4.5H_2O$	0.1 mg
H_3BO_4	0.1 mg
$Na_2MoO_4.2H_2O$	0.1 mg
$Na_2SeO_3.5H_2O$	0.1 mg
Nicotinic acid	2.5mg

Cyanocobalamin	2.5mg
p-aminobenzoic acid	2.5mg
Calcium pantothenate	2.5mg
Thiamine-hydrochloride	2.5mg
Riboflavin	2.5mg
Lipoic acid	2.5mg
Folic acid	0.1 mg
Biotin	0.1 mg
Pyridoxine-hydrochloride	0.1 mg
Yeast extract	5 mg
L-cystein	0.1 g

3.2 Chemicals, reagents, glassware and plastic ware

Analytical grade chemicals and reagents employed in the study were obtained from Hi-Media, Sigma-Aldrich, and Merck. Molecular grade chemicals and products were procured from Promega, and Thermo Scientific. The glassware used was purchased from Borosil, and the plasticware was purchased from Eppendorf and Tarsons.

3.3 Sterilization

Glasswares were sterilized at a temperature of 180ºC for one hour using hot air oven. All the culturing and production media were sterilized through autoclaving at 121°C, 15 psi for 20 min (minutes). All the microbiological techniques such as like culturing, inoculation, sampling etc., were performed under aseptic conditions using laminar air flow (LAF) chamber.

3.4 Isolation and screening of xylanolytic bacteria

3.4.1 Sampling and collection site

Hot springs are known to harbor diversity of potent thermophiles. Thus, in the present study Tattapni hot spring situated in Districtt. Mandi, Himachal Pradesh, India (Fig. 3.1) was selected as the site for sample collection. Soil sample was collected in sterile container and sealed. The sample was then stored at 4°C in laboratory before further processing.

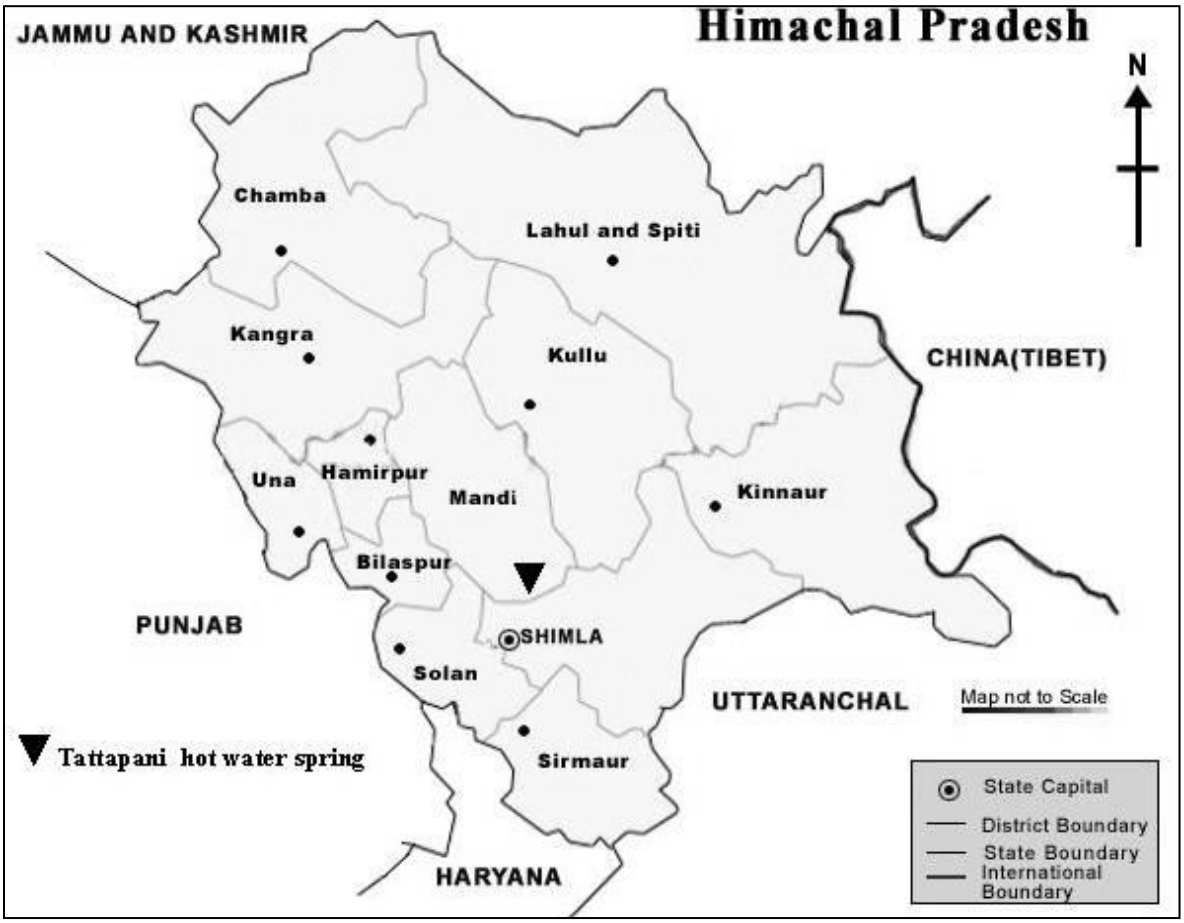

Figure 3.1: Location of Tattapani hot spring [5].

3.4.2 Media enrichment and isolation of thermophilic xylanolytic bacteria

For isolation of thermophilic xylanolytic bacteria from the hot spring soil sample; an enrichment culture technique was followed. For enrichment, one gram soil sample was inoculated into 100 mL minimal media (pH 7) contained in an Erlenmeyer flask. BW xylan (BW) at a concentration of 0.1%, w/v was an additional component in the minimal media; provided as the carbon source. The flask with enrichment culture was incubated at 60°C and 150 rpm of agitation rate. After 72 h, 1 mL of the enriched culture was inoculated into another flask with same media and incubation was carried out under same conditions for second enrichment. In similar way, four such sequential transfers were done to eventually get the culture enriched with xylanolytic thermophiles (Fig. 3.2). From the final enrichment flask, 1mL of culture was taken and serially diluted with distilled water. Different dilutions were spread on NA plates containing 0.1% BW

xylan. The xylan NA plates were then kept overnight at 60°C and the thermophilic bacterial colonies formed were observed next day.

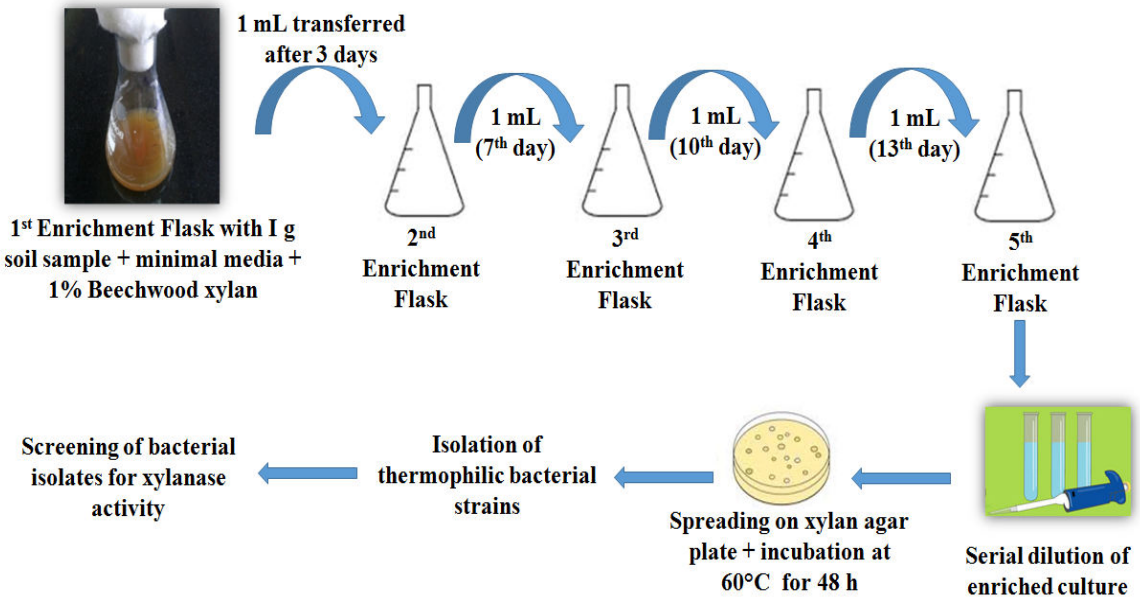

Figure 3.2: Diagrammatic representation of enrichment and isolation of thermophilic xylanolytic bacterial strains from hot spring soil sample.

3.4.3 Screening of thermophilic xylanolytic bacteria

The diverse thermophilic bacterial population obtained after the enrichment technique was screened for the potent strains with xylanase activity. For this primary and secondary screening was performed as follows:

3.4.3.1 Primary screening

It was done through Congo Red Assay [170]. Cultures of different thermophilic bacterial strains were grown in NB at 60°C with continuous agitation at 150 rpm. After 24 h, each culture was centrifuged and supernatant was collected. Wells were punctured on xylan NA plates containing 0.5% (w/v) BW xylan and filled with supernatant of different bacterial strains. The plates were then incubated for 48 h at 60°C; followed by staining with Congo red solution (0.1% Congo red dye in distilled water) and 15 min incubation at room temperature. Afterwards, plates were destained by flooding them with 1M NaCl and intermittent manual shaking. The washing with NaCl was ceased after the zone of hydrolysis was visible.

3.4.3.2 Secondary screening

BACTERIAL cultures were grown in minimal medium (pH 7; 0.1% w/v BW xylan) at 60°C with agitation at 150 rpm. After 48 h, each bacterial culture was centrifuged (10,000 rpm/10 min) to get supernatant for xylanase activity assay.

3.5 Xylanase activity assay

For activity assay bacterial culture supernatant was used as crude enzyme and 0.5% w/v BW xylan in SP buffer (100 mM, pH 7) was used as substrate. Reaction mixture of substrate (0.5 mL) and enzyme (0.5 mL) was given incubation in water bath for 5 min 70°C [171]. After incubation, the reaction was halted by adding 1.5 mL DNS and 10 min boiling. Afterwards the samples were cooled on ice for stabilization of the color produced. The optical absorbance of the xylose sugar produced was read at 540 nm. Xylanase unit activity was defined as "the amount of enzyme required to release 1 μmol of xylose-equivalent reducing groups per min under assay conditions".

3.6 Identification of xylanolytic bacterial isolate

Morphological characterization was done through Gram staining and KOH string test. To reveal the species identity, molecular characterization was practiced through 16S rRNA sequencing. For 16S rRNA gene amplification, genomic DNA of the isolate was extracted using PROMEGA DNA isolation kit. Universal primers 27F (5′-AGA GTT TGATCMTGG CTCAG, where M is A or C) and 1492R (5-ACC TTG TTA CGA CTT) were used for amplification under optimized conditions [172]. The sequencing of PCR product was performed by Xcelris Labs Ltd. Ahmedabad. The comparative and phylogenetic analysis of the sequence obtained was done using NCBI BLAST and MEGA 4, respectively [173].

3.7 Production of xylanase

Exploiting the xylanolyitc bacterial isolate, xylanase production was carried out. Minimal medium with 0.5% (w/v) BW xylan was used as the production medium. Overnight grown bacterial culture was inoculated (1% v/v) into 100mL production medium; contained in 500 mL Erlenmeyer flask. Conditions for xylanase production were 60°C temperature, 48 h incubation time and agitation speed rate of 150 rpm. Afterwards the production medium was centrifuged

(10,000 rpm/10 min) and supernatant and cell pellet were collected for assaying extracellular and intracellular xylanase activity, respectively.

3.8 Localization and induction of xylanase

In order to localize the production of xylanase, its extracellular and intracellular activity was estimated. For detecting the extracellular activity, xylanase activity assay was peformed with supernatant of the production medium. For estimating intracellular xylanase activity, the cell pellet was subjected to ultrasonication. The cell debris (settled at the bottom) obtained after ultrasonication were discarded, whereas the cell lysate was assayed by DNS method for xylanase activity. To analyze inducible nature of xylanase, its production was carried out at above mentioned conditions by using the production medium (50 mL, pH 7) containing xylose (0.1% w/v) as carbon substrate in place of BW xylan. Control of the experiment was xylanase production being carried out under same conditions with BW xylan (0.1% w/v) as carbon source. Production was followed by activity assay for xylanase.

3.9 Parametric optimization of xylanase production by OFAT

Optimization is an imperative process for attaining maximum xylanase production under given physio-chemical conditions. OFAT method was followed for optimization of parameters influencing xylanase production. Using this method, one variable is optimized at a time and that optimized value is inculcated in the succeeding step. Various parameters such asinoculum size (0.5-2%, v/v), temperature (40-60°C), rate of agitation speed (50-200 rpm), pH (4-10), incubation time (24-120 h), nitrogen sources: peptone, urea and beef extract (0.005 g/L), nitrogen concentration (0.005-5 g/L) and substrate concentration (0.5-3%) were optimized. Also, targeting the cost-effective production of xylanase, abundantly available cheap substrates such as wheat straw, wheat bran, corn cob and rice straw were valorized for xylanase production under the optimized conditions. Optimization study was initiated by using 1% (v/v) inoculum (~3.7×10^7CFU/mL) for xylanase production under SmF for 48 h at 60°C and 150 rpm. After that, production medium was centrifuged (10,000 rpm/10 min) and subjected to xylanase activity assay.

3.10 Statistical optimization through response surface methodology

The significant parameters in terms of having maximum influence on the xylanase activity were selected from the OFAT optimization. The selected parameters were then subjected to statistical optimization (RSM) to study the mutual effect of the parameters on xylanase production. CCD was opted for performing RSM optimization with selected influential parameters. Each parameter was analyzed at five coded level -α, -1, 0, +1 and +α (Table 3.2). Under CCD, experimental trials were generated, using Design Expert 10 software. The experimental trials comprised of axial points, factorial points and central point.

Table 3.2: Range of variables at different levels used in central composite design.

Independent variables	**Units**	**Range and Level**				
		-α	-1	0	+1	+α
A: Temperature	degree Celsius	29.8	40	55	70	80.2
B: Incubation time	hours	12.7	40	80	120	147.3
C: Agitation	rpm	65.9	100	150	200	234.1

During statistical optimization, value of only selected parameters was varied; while media components (minimal medium) and other optimized parameters were constant. ANOVA was used for statistical analysis and data interpretation and plotting of response surfaces was done using Design Expert 10 software.

3.11 Model validation

Validation of the RSM model was done by carrying out xylanase production under the optimum conditions predicted by the model for maximum response.

3.12 Acessory cellulases and hemicellulases

Filter paper activity (FPase) and endo-1,4-β-D-glucanase (EC 3.2.1.4) activity was assayed for complete cellulase activity [174]. For FPase activity, crude GTX1 xylanase was appropriately diluted with Tris-HCl (pH 8, 50mM) to make 1mL reaction mixture. Afterwards, whatman filter paper no. 1 (1.0×6.0 cm) was immersed in the mixture and was given incubation for for 1 h at 60°C; followed by DNS method for sugar estimation. Filter paper unit (FPU) activity was defined as "the amount of enzyme releasing 1 μmol of reducing sugar from filter paper per mL per min". Endoglucanse activity was determined by 30 min incubation of 0.5 mL crude xylanase with 0.5 mL of 1% (w/v) carboxymethylcellulose (pH 6, 50 mM SP) at 60°C. Reducing sugars produced were determined by DNS method. Endoglucanase unit activity was defined as "the amount of enzyme releasing 1 μmol glucose per min under the specified assay conditions". For assay of EC 3.2.1.91; exo-1,4-β-D-glucanase, EC 3.2.1.21; β-D-glucosidase, β-D-xylosidase, α-L-arabinofuranosidase, feruloyl esterase and acetyl xylanesterase activities were assayed using substrates (3mM) namely pNPC, pNPG, pNPX, pNPA, pNP ferulate and pNP acetate for microtiter plate assay. Activity assay for all enzymes was carried out for 30 min at 60°C with the reaction mixture of diluted enzyme (25 μL), pNP substrate (25 μL) and 50 mM SP buffer of pH 6 (50 μL). The reaction was terminated by addition of 100 μL glycine-NaOH buffer (0.4 M, pH 10.8) [175]. The color of released para nitro-phenol (pNP) was read at 405 nm. One unit of enzyme activity was defined as "the amount of enzyme capable of releasing 1 μmol pNP from the pNP linked substrates per min".

3.13 Characterization of GTX1 xylanase

pH optima of GTX1 xylanase activity was estimated at diverse pH range of 3-10. Different buffers were used for respective pH range; at a concentration of 100mM. For pH 3–5; sodium citrate, pH 6–7; sodium phosphate, pH 8-9; Tris-HCl and for pH10; glycine–NaOH buffer was used. For determining the optimum temperature, activity assay was performed at varying temperatures of 30°C to 100°C at an optimum pH. Thermostability of the GTX1 crude xylanase was estimated at 60°C and 70°C through its pre-incubation for 4h in 100 mM Tris-HCl (pH 8). It

was followed by determination of relative xylanase activity at standard assay conditions for xylanase mentioned before. A control was run in parallel without any pre-incubation. For studying the pH stability, the crude enzyme was pre-incubated for 4h at 60°C in different pH buffers (3-10) whereas crude xylanase without pre-incubation was referred as a control. After that, estimation of relative xylanase activity was done by the standard DNS method. Effect of various metal ions was studied by preincubating the crude xylanase with a metal ion (10mM) at 60°C for 4 h; no metal ion was added in control. Afterwards relative activity was estimated under standard conditions.

Hydrolysis profile of BW xylan was analyzed by incubating 20 U xylanase/g xylan [17] in 100mM Tris HCl buffer (pH 8) at 60°C with agitation at 150 rpm for 48 h. No xylanase was added in the control for the experiment. After 48 h, products from hydrolysis were analyzed through HPLC using RI detector and Aminex HPX-87P column (300 X 7.8 mm; column). 20 µL of sample was injected using Milli-Q water as the mobile phase and the analysis was done at column temperature of 80°C with 0.6 mL/min flow rate.

Gene length and molecular weight of xylanase from *Geobacillus thermodenitrificans* X1 (GTX1) was also determined. Reverse and forward primers were designed using Primer 3 tool for amplification of xylanase gene from GTX1. For molecular weight determination, zymogram of GTX1 xylanase was performed. For, zymogram crude xylanase was concentrated using Amicon Ultra-15-Millipore with a cut off of 10 kDa in the membrane. Concentrated GTX1 xylanase was treated with SDS sample buffer; followed by heating for 5 min at 95°C, prior to loading on gel. Sample were run on 12% SDS PAGE gel, followed by cutting the gel such that the lane containing protein ladder is separated from the gel containing crude xylanase. The ladder containing gel was stained with coomassie dye. On the other hand, for zymogram the gel with crude xylanase was given three successive washings of 20 min each with 20% isopropanol. After that gel was immersed for 1 h incubation at 60°C, in SP buffer (50 mM, pH 7) containing 1% BW xylan; followed by congo red assay.

3.14 Immobilization of xylanase

3.14.1 Preparation of Xy-CLEAs

For precipitating xylanase molecules; chilled acetone (100%, v/v) was added as precipitating agent into 1.67 mg/mL of crude GTX1 xylanase (10 mL) (Fig. 3.3). The process was continued

for 2 h at 4°C with continuous stirring at 150 rpm. Afterwards, glutaraldehyde (25% v/v) was added as cross-linker at 0.02% (v/v) final concentration for the formation of Xy-CLEAs. The cross-linking process was conducted for 4 h at 4°C and continuous agitation (150 rpm). For recovery of CLEAs, the reaction mixture was centrifuged (10,000 rpm; 5 min) at 4°C. After that, three times washing was given to the CLEAs using SP buffer (50 mM, pH 7) for removing unbound xylanase molecules and residual GA [39]. CLEAs were resuspended in the same buffer (50 mM; pH 7); followed by activity assay for xylanase.

3.14.2 Optimization of parameters affecting preparation of Xy-CLEAs

For recovery of maximum activity of Xy-CLEAs, influencing parameters need to be optimized. Therefore, for preparation of Xy-CLEAs; various parameters comprising of protein concentration (0.8-3.3 mg/mL), type of precipitant, protein: precipitant ratio (1:5-1:25), precipitation time (30-150 min), cross-linker concentration (0.005-0.05% v/v) and reaction time (2-10 h) were optimized by OFAT method. Relative xylanase activity was estimated for analyzing the effect of these parameters.

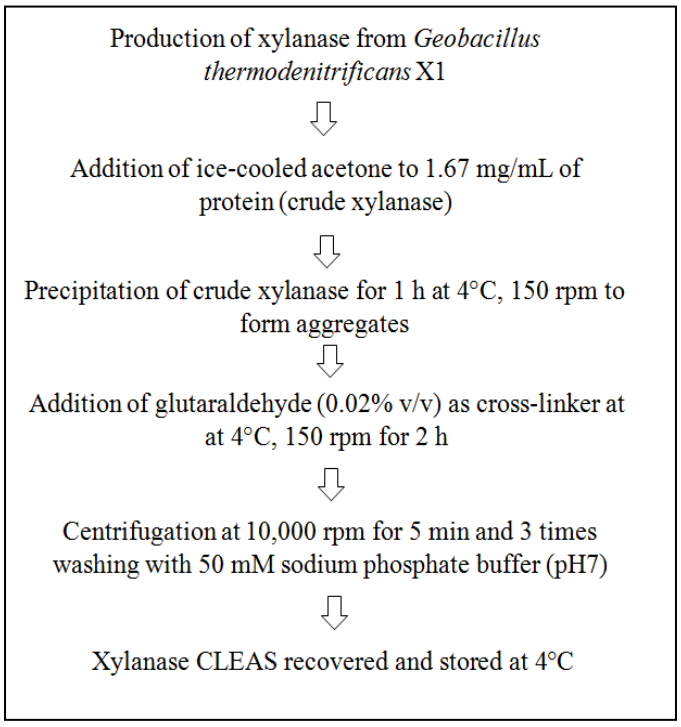

Figure 3.3: The schematic representation of preparation of thermosatble xylanase CLEAs from *Geobacillus thermodenitrificans* X1.

3.14.3 Characterization of Xy-CLEAs

3.14.3.1 Morphological analysis

Morphology of Xy-CLEAs (lyophilized) was studied by SEM analysis using FEI (Thermo Fisher) Nova Nano SEM-450. For determining CLEA particle radius, Dynamic light scattering (DLS) analysis was performed at room temperature using Malvern Zeta Sizer. An average of three runs was considered for estimation of the size of Xy-CLEAs.

3.14.3.2 Chemical characterization

Amendments in the structure of protein after formation of Xy-CLEA were determined through FTIR analysis performed by following KBr pellet method [14]. Transmittance a spectrum (wave number range of 4000 cm-1 to 500 cm-1) of free xylanase was recorded as a control for comparative analysis with respect to spectra peaks of Xy-CLEAs.

3.14.3.3 Biochemical characterization

3.14.3.3.1 Optimum pH and temperature

pH optima for activity of free and immobilized GTX1 xylanase was estimated at varied pH range of pH 3-10. Different buffers were used for respective pH range; at a concentration of 100mM. For pH 3–5; sodium citrate, pH 6–7; sodium phosphate, pH 8-9; Tris-HCl and for pH10; glycine–NaOH buffer was used. For determining the optimum temperature, activity assay was performed at varying temperatures of 30°C to 100°C at pH 7 (100 mM SP).

3.14.3.3.2 Thermostability and pH stability

For estimation of thermostability, free GTX1 xylanase and its CLEAs were pre-incubated for 4 h at 60°C and 70°C in Tris-HCl (pH 8; 100 mM); followed by estimation of relative xylanase activity at standard assay conditions. Control reaction mixture (100% relative activity) was not given any incubation. Thermal inactivation was calculated as per the equation: $\ln A/A_0 = K_d t$, where

A_0: initial activity without pre-incubation

A: residual activity after thermal exposure

K_d: thermal inactivation rate constant (h^{-1})

t: exposure time (h).

Half-life ($t_{1/2}$, h) was calculated as per the equation: $t_{1/2}=\ln 2/K_d$. pH stability was studied by incubating free and immobilized xylanase for 4 h at 60°C in different pH range (3-10) whereas xylanase without incubation was the control; considered to have 100% relative activity.

3.14.3.3.3 Reusability and storage stability

Xy-CLEAs were stored for 8 weeks at 4°C in SP buffer (pH 7; 50 mM). After every week, relative xylanase activity (%) was measured with respect to initial activity (100%) referred as control. Reusability was estimated by reusing Xy-CLEAs for repetitive six cycles of xylanase assay. Supernatant was subjected to xylanase assay whereas CLEAs in the pellet were given two times washings with SP buffer (50 mM; pH 7) before being reused. Relative activity of the first cycle was considered as 100%. After completion of last cycle; CLEAs were washed and lyophilized for SEM analysis to observe morphological changes after consecutive usage.

3.15 Application of crude GTX1 xylanase for paper pulp bio-bleaching

3.15.1 Pretreatment of pulp with crude xylanase

The agropulp (blend of 85% wheat straw and 15% bagasse) was collected from Kauntum Papers Limited situated in district Hoshiarpur, Punjab. The agropulp was distributed into three parts (100 g each) comprising of two test samples (T1 and T2) and one control sample (Fig. 3.4). Each Test sample was pretreated with xylanase dosage of 50 U/mL per one gram pulp; followed by incubation at 70°C for a retention period of 90 min with intermittent kneading. In contrast, the control sample (100 g) was not given any xylanase pretreatment.

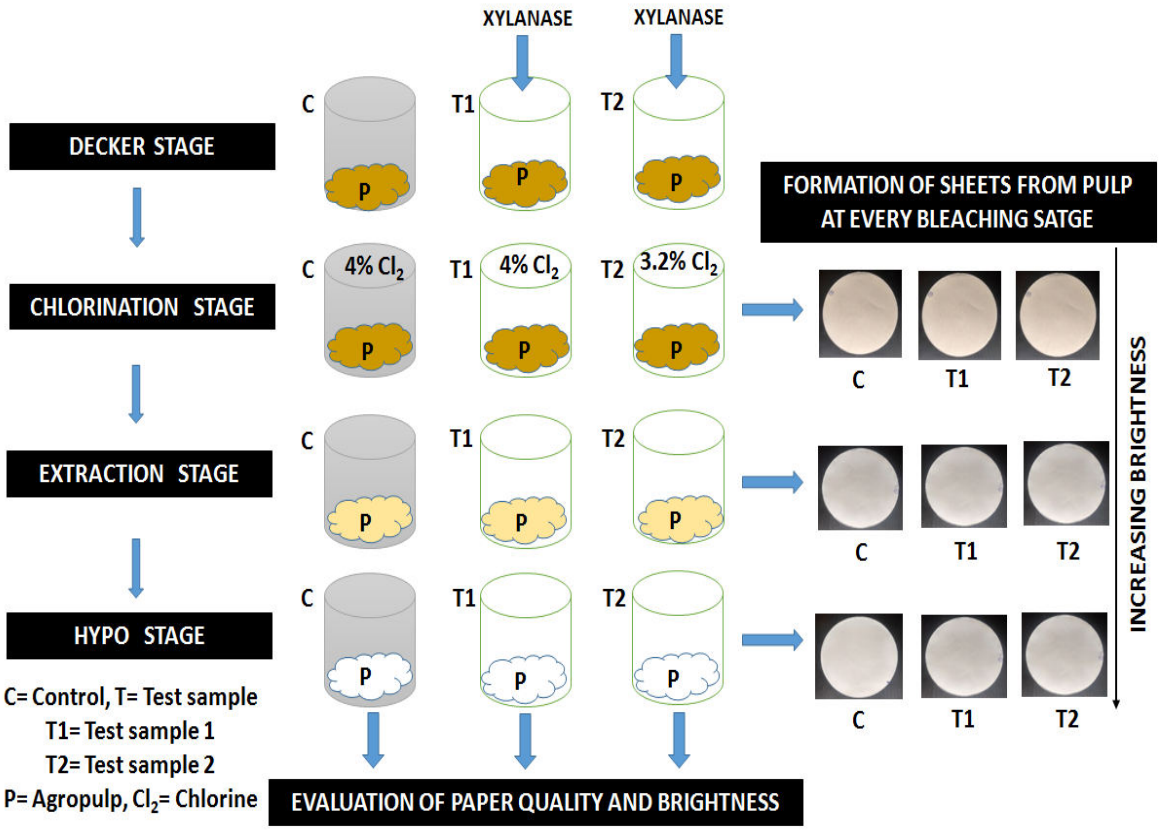

Figure 3.4: Diagrammatic representation of paper pulp bio-bleaching by xylanase.

After incubation, pulp from test and the control sample was examined for pH, kappa no. and brightness, followed by washing with fresh water. It was calculated by Tappi test method T 236 om-85. Brightness was determined using CM-3630 spectrophotometer (Konica Minolta) at 457 nm following Tappi Test method T 452 om-87. The washed pulp was appropriately filtered before the succeeding bleaching stages: Chlorination, Alkali Extraction and Hypo stage.

3.15.2 Chlorination stage

For chlorination, the pulp consistency was maintained at 3%. The chlorine dosage was varied among the pulp samples. T1 and the control sample were treated with 4% chlorine while T2 sample was treated with 3.2% chlorine. This 20% chlorine reduction for T2 sample was done to evaluate the potential of xylanase to reduce the chlorine consumption in the bleaching process. Chlorination process was carried out at ambient temperature for 45 min, followed by washing with fresh water. Afterwards, pulp was screened for brightness, initial and final pH. A fraction of pulp from all the samples (T1, T2 and control) was pressed into sheets using automatic sheet making machine and dried by sheet dryer.

3.15.3 Extraction stage

Chlorinated pulp was treated with 2% caustic soda and hydrogen peroxide (1.32%) at a consistency of 10%. It was then followed by examination of initial pH and incubation at 70°C for 120 min. Afterwards pulp was given washing with excess of fresh water and filtered for further analysis. Kappa no. and brightness of the pulp was determined after extraction stage and also sheets were prepared.

3.15.4 Hypo stage

The pulp (consistency 10.5%) was given treatment of sodium hypochlorite solution (available chlorine 2.5%) and 0.1% sulphamic acid for 120 min at 45°C. Pulp was examined for changes in brightness and pH before and after treatment. Afterwards, sheets were prepared and examined for comparative analysis of the properties of the paper from different samples (T1, T2 and control).

3.15.5 Properties of paper

Paper sheets prepared from all samples were evaluated for quality properties in terms of viscosity, basis weight, bulk weight, breaking length, tear factor, and burst factor. Freeness measures the drainability of pulp suspension. Viscosity was measured using Viscometer (Cannon fenske) according to Tappi test method T 230 om-82. Bulk and basis weight were determined to evaluate the print quality and heaviness of paper, respectively. Bulk indicates thickness or volume in relation to weight. Basis weight gives the weight per unit area and thus decides that how much area of paper the customer will get for given weight. For determining the strength of paper, properties such as breaking length, tear factor, and burst factor were assessed. Breaking length was analyzed by following Tappi test method T 452 om-87. Tear factor was calculated using internal tearing resistance tester (Fibretic instrument Roorkee, 247667) following Tappi test method T 414. It is defined as the maximum force required tearing a paper in a direction perpendicular to the direction of stress (tearing tester). Bursting factor of paper was determined by using digital bursting strength tester (in compliance with ISO 9001). This factor determines the stress that can be tolerated by paper before it ruptures when an external pressure is applied to it.